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(54) Title: COMPOSITIONS AND METHODS FOR RAPID ISOLATION OF PLASMID DNA

(57) Abstract

A composition for isolating plasmid DNA from a bacterial culture is disclosed, the composition containing at least about 3 M magnesium chloride and about 10 % (w/v) polyethylene glycol. A method of differentially precipitating plasmid DNA from a bacterial lysate is also disclosed, involving the steps of lysing bacterial cells to obtain a bacterial lysate; mixing magnesium chloride and polyethylene chloride with the lysate to form a mixture containing at least about 1 M magnesium chloride and about 3.3 % (w/v) polyethylene glycol, thereby precipitating cellular debris, chromosomal DNA, cellular RNA, and proteins; separating and removing the precipitated material to form a cleared lysate; mixing magnesium chloride and polyethylene glycol with the cleared lysate to form a mixture containing at least about 1.5 M magnesium chloride and about 5 % (w/v) polyethylene chloride, thereby precipitating plasmid DNA; and separating the precipitated plasmid DNA from the mixture, thereby obtaining isolated plasmid DNA. Another composition according to the invention contains an enzyme for digestion of cellular RNA, a stabilizer for protecting plasmid DNA from nuclease digestion, and a precipitating agent for precipitating plasmid DNA. Another method of isolating plasmid DNA from a bacterial culture comprises simultaneously digesting cellular RNA in a clarified lysate with an enzyme, protecting plasmid DNA from nuclease digestion, and precipitating plasmid DNA.

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COMPOSITIONS AND METHODS FOR RAPID ISOLATION OF PLASMID DNA

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND OF THE INVENTION

This invention relates to compositions and methods of use thereof for rapid isolation of plasmid DNA from bacterial cultures. More particularly, in one preferred embodiment the invention relates to a composition that can be used for preparing a cleared lysate by precipitating cellular debris, chromosomal DNA, cellular RNA, and proteins, and then can be used for differentially precipitating plasmid DNA from the cleared lysate. In another preferred embodiment, the invention relates to a composition comprising an enzyme for digesting cellular RNA, a stabilizer for protecting plasmid DNA from nuclease digestion, and a precipitating agent for precipitating plasmid DNA. A method of using this composition comprises effecting simultaneous digestion of cellular RNA, protection of plasmid DNA from nuclease digestion, and precipitation of plasmid DNA from a cleared bacterial lysate.

Isolation of plasmid DNA from bacterial cultures is a routine procedure in molecular biology. Over the years, many plasmid DNA isolation procedures have been developed, such as (a) SDS-NaOH lysis/phenol-chloroform extraction/ethanol precipitation, H.C. Birnboim & J. Doly, A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA, 7 Nucl. Acids Res. 1513-23 (1979); (b) boiling lysis/ethanol precipitation, D.S. Holmes & M. Quigley, A Rapid Boiling Method for the Preparation of Bacterial Plasmid, 114 Anal. Biochem.

193-97 (1981); (c) phenol-chloroform lysis and extraction/ethanol precipitation, S. Anant & K.N. Subramanian, Isolation of Low Molecular Weight DNA from Bacteria and Animal Cells, 216 Meth. Enzymol. 20-29 (1992); (d) SDS-NaOH lysis/ethanol precipitation/polyethylene glycol (PEG) purification, J. Sambrook et al., Purification of Plasmid DNA by Precipitation with Polyethylene Glycol, in Sambrook et al., Molecular Cloning 1.40-1.41 (2d ed., 1989); (e) SDS-NaOH lysis/glass or silica absorption, M.A. Marko et al., A Procedure for the Large Scale Isolation of Highly Purified Plasmid DNA using Alkali Extraction and Binding to Glass Powder, 121 Anal. Biochem. 382-87 (1982); and (f) SDS-NaOH lysis/CsCl gradient purification, J. Sambrook et al., Purification of Closed Circular DNA by Equilibrium Centrifugation in CsCl-EthBr Gradients, in J. Sambrook et al., Molecular Cloning 1.42-1.43 (2d ed., 1989). Many of these methods are quite reliable and some have been developed into commercial DNA isolation kits. However, they all have one or more shortcomings, such as being time consuming, requiring use of toxic chemicals, resulting in contamination with RNA, or requiring dedicated equipment. Since the isolation of plasmid DNA is a precedent step for many other steps in molecular cloning, a simple and rapid plasmid DNA isolation method is in continuing demand. Therefore, great effort has been and is being made to simplify plasmid isolation procedures by many investigators.

In view of the foregoing, is will be appreciated that providing compositions and methods of use thereof for rapid isolation of high yields of plasmid DNA from bacterial cultures wherein the plasmid DNA is uncontaminated with RNA and the method does not involve toxic chemicals or dedicated equipment would be a significant advancement in the art.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide a composition for rapid isolation of highly pure plasmid DNA from bacterial cultures.

It is also an object of the invention to provide a method of rapidly isolating highly pure plasmid DNA from bacterial cultures.

It is another object of the invention to provide a method of rapidly isolating plasmid DNA from bacterial cultures such that toxic chemicals or dedicated equipment are not required.

It is still another object of the invention to provide a method of isolating plasmid DNA from bacterial cultures that is adaptable to large scale, miniprep, and 96-well microtiter plate formats.

These and other objects can be achieved by providing a composition comprising at least about 3 M $MgCl_2$ and about 10% (w/v) of polyethylene glycol. Preferably, the polyethylene glycol has an average molecular weight of about 6000 to about 8000, and the $MgCl_2$ concentration is about 3 molar.

A method of isolating plasmid DNA from a bacterial culture comprises the steps of:

(a) lysing bacterial cells in the bacterial culture to result in a bacterial lysate;

(b) mixing $MgCl_2$ and polyethylene glycol with the bacterial lysate to form a first mixture comprising at least about 1 M $MgCl_2$ and about 3.3% (w/v) polyethylene glycol, thereby precipitating cellular debris, chromosomal DNA, cellular RNA, and proteins in the first mixture;

(c) separating and removing precipitated cellular debris, chromosomal DNA, cellular RNA, and proteins from the first mixture to form a cleared lysate;

(d) mixing $MgCl_2$ and polyethylene glycol with the cleared lysate to form a second mixture comprising at least about 1.5 M $MgCl_2$ and about 5% (w/v) polyethylene

glycol, thereby precipitating plasmid DNA in the second mixture; and

(e) separating precipitated plasmid DNA from the second mixture to result in isolated plasmid DNA.

Preferably, the bacterial cells are lysed by alkaline lysis. It is also preferred that the $MgCl_2$ and polyethylene glycol are contained in a composition comprising at least about 3 M $MgCl_2$ and about 10% (w/v) polyethylene glycol. More preferably, the composition comprises about 3 M $MgCl_2$, and the polyethylene glycol has an average molecular weight of about 8000.

A method of making a cleared bacterial lysate from a bacterial culture comprises the steps of:

(a) lysing bacterial cells in the bacterial culture to result in a bacterial lysate;

(b) mixing $MgCl_2$ and polyethylene glycol with the bacterial lysate to form a first mixture comprising at least about 1 M $MgCl_2$ and about 3.3% (w/v) polyethylene glycol, thereby precipitating cellular debris, chromosomal DNA, cellular RNA, and proteins; and

(c) separating precipitated cellular debris, chromosomal DNA, cellular RNA, and proteins from the first mixture to form the cleared lysate.

In another illustrative embodiment of the invention, a composition for isolating plasmid DNA from bacterial cultures comprises an effective amount of an enzyme for digesting cellular RNA, an effective amount of a stabilizer for protecting plasmid DNA from nuclease digestion, and an effective amount of a precipitating agent for precipitating plasmid DNA. Preferably, the enzyme is ribonuclease and the effective amount of enzyme is in the range of about 1-100 μg/ml, more preferably 5-50 μg/ml. It is also preferred that the stabilizer is a bivalent cation chelating agent, more preferably ethylenediaminetetraacetic acid (EDTA) or salts thereof, and the effective amount thereof is in the range of about 1-100 mM, more preferably 5-50 mM.

It is further preferred that the precipitating agent is polyethylene glycol and the effective amount of precipitating agent is in the range of about 5-25% by weight. The polyethylene glycol preferably has an average molecular weight of about 6000-8000.

A method of isolating plasmid DNA from a bacterial culture comprises:

(a) concentrating bacterial cells from a bacterial culture to obtain a pellet;

(b) resuspending the bacterial cells in the pellet in a selected volume of an aqueous solution to obtain a concentrated cell suspension;

(c) lysing the bacterial cells in the concentrated cell suspension to obtain a lysed cell suspension;

(d) precipitating cell debris in the lysed cell suspension and separating the precipitated cell debris from the lysed cell suspension to result in a clarified cell lysate;

(e) mixing the clarified cell lysate with an effective amount of a composition comprising an effective amount of an enzyme for digesting cellular RNA, an effective amount of a stabilizer for protecting plasmid DNA from nuclease degradation, and an effective amount of a precipitating agent for precipitating plasmid DNA, to obtain a mixture, and incubating the mixture for a time and at a temperature sufficient for the enzyme to digest cellular RNA contained in the mixture and for the precipitating agent to precipitate plasmid DNA contained in the mixture;

(f) separating the precipitated plasmid DNA from the mixture and washing the precipitated plasmid DNA in a wash solution to obtain a DNA pellet; and

(g) drying the DNA pellet and then resuspending the DNA pellet in a selected amount of aqueous medium to result in isolated plasmid DNA.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 shows a representation of minipreps of 1:1 serial dilutions of pUC19 prepared according to the present invention (lanes 1, 3, 5, and 7) and by ethanol precipitation (lanes 2, 4, 6, and 8) from the same cell lysate after separation in 1.0% agarose gel and visualization by ethidium bromide staining: lane M - BstEII/1 DNA molecular weight ladder.

FIG. 2 shows a representation of restriction endonuclease fragments of plasmid pBKBH10S (11857 bp) digested with 32 restriction enzymes separated on 0.6% agarose gel and stained with ethidium bromide: 0-uncut; 1-BsaHI; 2-NotI; 3-SmaI; 4-XbaI; 5-BamHI; 6-BpmI; 7-BspHI; 8-ClaI; 9-DrdI; 10-NdeI; 11-PstI; 12-SacI; 13-SalI; 14-SapI; 15-XhoI; 16-EcoRI; 17-EcoRV; 18-BsgI; 19-HaeII; 20-HindIII; 21-KpnI; 22-XmnI; 23-AlwNI; 24-BsbI; 25-BglII; 26-SspI; 27-ScaI; 28-AccI; 29-StyI; 30-DraI; 31-TaqI; and 32-HphI.

FIG. 3 shows a representation of plasmid DNA prepared in a microtiter plate procedure according to the present invention wherein the plasmid DNA from columns 9-12 was separated on 0.8% agarose gel and stained with ethidium bromide; columns 10 and 12 were not inoculated with bacteria, and no plasmid DNA was found in the uninoculated wells.

DETAILED DESCRIPTION

Before the present compositions and methods for isolating plasmid DNA from a bacterial culture are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope

of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, "TE buffer" comprises 10 mM Tris, 1 mM EDTA, pH 8.0.

As used herein, "alkaline lysis solution" comprises 1% SDS in 0.2 N NaOH.

As used herein, "effective amount" means an amount sufficient to achieve a selected response without undue adverse effects. For example, an effective amount of an enzyme for digesting cellular RNA is an amount sufficient to digest such cellular RNA at a selected temperature within a selected period of time. An effective amount of stabilizer is an amount sufficient to protect plasmid DNA from nuclease digestion at a selected temperature for a selected amount of time. An effective amount of a precipitating agent is an amount sufficient to precipitate a selected plasmid at a selected temperature in a selected amount of time. According to the guidance provided herein and what is well known in the art, such effective amounts can be determined by a person skilled in the art without undue experimentation.

As used herein, "enzyme for digesting cellular RNA" and similar terms mean a nuclease for digesting such cellular RNA. Preferably, such nuclease is ribonuclease A. Preferably, ribonuclease A is contained in the claimed composition in an amount ranging from about 1 to about 100 μl/ml.

As used herein, "stabilizer" means an agent for protecting plasmid DNA from nuclease digestion.

Preferred stabilizers are bivalent cation chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and salts thereof. Preferred amounts of stabilizers, such as EDTA or salts thereof, in the presently claimed composition are in the range of about 1 to about 100 mM.

As used herein, "precipitating agent" means a polymer, such as polyethylene glycol or dextran, for precipitating plasmid DNA from solution. Polyethylene glycol is especially preferred, and is generally used in concentrations of about 5-50% by weight in the presently claimed composition. It is also preferred to use polyethylene glycol (PEG) polymers that have a relatively low average molecular weight, such as PEG 6000 or PEG 8000.

One illustrative embodiment of the present composition comprises an aqueous solution of at least about 3 M $MgCl_2$ and about 10% (w/v) of polyethylene glycol, preferably having an average molecular weight of about 8000 (PEG8000). The concentration of the PEG8000 is fairly critical to the proper functioning of the composition, but the concentration of the $MgCl_2$ can vary. The optimal concentration of $MgCl_2$ has been determined to be about 3 molar. The $MgCl_2$ and polyethylene glycol can be added to the cell lysate to obtain a cleared lysate. Subsequently, additional $MgCl_2$ and polyethylene glycol can be added to the cleared lysate to precipitate plasmid DNA. The $MgCl_2$ and polyethylene glycol can be added either as a mixture or sequentially as separate solutions, but a mixture of the $MgCl_2$ and polyethylene glycol is preferred because of ease of preparation and use.

An illustrative method of using the present composition for differentially precipitating plasmid DNA from a bacterial culture comprises lysing the cells in a bacterial culture, preferably with alkaline lysis solution as is well known in the art; adding about 0.5 volume of the $MgCl_2$/PEG8000 composition and mixing to

precipitate cellular debris, RNA, chromosomal DNA, and protein and removing the precipitated cellular debris, RNA, chromosomal DNA, and protein to result in a cleared lysate; mixing about the same volume of the $MgCl_2$/PEG8000 composition used in the previous step with the cleared lysate to differentially precipitate the plasmid DNA; and recovering the precipitated plasmid DNA. It is preferred to wash the precipitated plasmid DNA with a wash solution, such as with 70% ethanol as is well known in the art. The resulting plasmid DNA is pure enough for being digested with a restriction endonuclease or for nucleotide sequencing applications.

The present composition and method can be used for purifying plasmid DNA from cell lysates and also for the following applications: (1) as a pre-purification step in preparing ultra-pure plasmid DNA by anion-exchange or other chromatographic methods; (2) to isolate and purify other episomal DNA molecules, such as viral DNAs from various cells and tissues, such as from mammalian cells; and (3) as a post-reaction cleanup step to remove or recover other molecules, such as RNA, single-stranded DNA, nucleotides, enzymes, and the like from double-stranded DNA.

The present invention is an improvement over a procedure by Toa Gosei Chemical Industrial Ltd., JP 61-4719, pp. 533-36 (1986), and similar procedures, M. Hattori & Y. Sakaki, Dideoxy Sequencing Method Using Denatured Plasmid Templates, 152, Anal. Biochem. 232-38 (1986); K.D. Cole, Purification of Plasmid and High Molecular Mass DNA Using PEG-salt Two-phase Extraction, 11 BioTechniques 18-24 (1991), that use PEG to isolate and purify plasmid DNA. In these methods, the cell lysate is treated with phenol-chloroform to extract cellular protein, the DNA and RNA in the lysate are precipitated with ethanol, the RNA is then digested with RNase at 37°C for 30 minutes, and finally the plasmid DNA is isolated by PEG precipitation.

A method for the preparation of large numbers of "mini" plasmid preparations, i.e. "minipreps," represents a significant contribution to biotechnology. In many gene sequencing projects, such as the Human Genome Project, millions of plasmid minipreps are prepared annually, thus representing a time-consuming and rate-limiting step in these studies. By preparing plasmid minipreps in a 96-well microtiter plate according to the present invention, the process of cell culture through DNA sequencing is streamlined, since automated DNA sequencing reactions in a 96-well format is now available. N. Avdalovic, Automation of DNA Sequencing Protocols Using Fluorescent Primers and the Beckman Biomek 1000 Workstation, Beckman Application Notes ROB-AN-015, pp. 1-14 (1989).

Presently, the only 96-well plate miniprep technology is available from Promega Corporation. D. Romanin et al., Use of a 96 Well Format for Wizard Miniprep DNA Isolations, 49 Promega Notes 28-32 (1994). The Promega method is based on the binding of DNA to a silica resin, which involves the transfer of cell lysates to a set of mini-columns, addition of a DNA binding resin to the lysates, washing the samples in a manifold, and elution of the plasmid DNA from the columns into a 96-well plate. The presently claimed method is not only based on a different DNA isolation mechanism, but also involves different DNA isolation procedures. Unlike Promega's method, the present method does not require dedicated equipment or accessories, and all the steps are performed in the 96-well plates, from cell culture through isolating the plasmid DNA.

Example 1

An illustrative composition according to the present invention for use in isolating plasmid DNA from a bacterial culture was prepared by dissolving $MgCl_2$ and

PEG8000 in deionized water to concentrations of 3 M $MgCl_2$ and 10% (w/v) PEG 8000.

Example 2

In this example, a procedure for using the composition according to Example 1 for isolating plasmid DNA is described:

1. Pellet 1.5 ml of an overnight bacterial culture by sedimentation at 12,000 x *g* for about 1-60 seconds.

2. Discard the culture medium, add about 100 μl of deionized water to the cell pellet, and vortex to resuspend the cells.

3. Add 100 μl of alkaline lysis solution comprising 1% SDS and 0.2 N NaOH to the cell suspension, and invert the tube to mix, resulting in a bacterial lysate.

4. Add 100 μl of the solution of Example 1, invert the tube to mix, and centrifuge at 12,000 x *g* for 1 minute to pellet the cell debris, chromosomal DNA, cellular RNA and protein to obtain a cleared lysate.

5. Transfer the cleared lysate to a tube pre-loaded with 100 μl of the solution of Example 1, vortex to mix, and centrifuge at 12,000 x *g* for 10 minutes to pellet the plasmid DNA.

6. Discard the supernate, rinse the plasmid DNA pellet with 70% ethanol, dry the DNA pellet, and then suspend it in 50 μl of TE buffer.

Example 3

In this procedure, a mid-sized preparation, i.e. "midiprep," procedure using the composition according to Example 1 is described:

1. Pellet 50 ml of overnight bacterial culture at 12,000 x *g* for 60 seconds.

2. Suspend the pellet in 1 ml of deionized water by pipetting.

3. Add 3 ml of 1% SDS, 0.2 N NaOH, and invert the tube to mix, thus obtaining a bacterial lysate.

4. Add 2 ml of the solution of Example 1, and invert the tube to mix.

5. Centrifuge the tube at 12,000 x *g* for 5 minutes to pellet the cell debris, chromosomal DNA, cellular RNA, and protein, thus obtaining a cleared lysate.

6. Transfer the cleared lysate to a fresh tube.

7. Add 2 ml of the solution of Example 1, mix by vortexing, and incubate the tube and its contents at 37°C for 5 minutes.

8. Centrifuge at 12,000 x *g* for 10 minutes to pellet the plasmid DNA, and decant and discard the supernate.

9. Wash the plasmid DNA pellet twice by suspending it in 5 ml of 70% ethanol, centrifuging the pellet to the bottom of the tube, and then decanting the supernatant.

10. Dry the plasmid DNA pellet, and then resuspend it in 500 μl of TE.

Example 4

In this example, a large-scale miniprep procedure using 96-well microtiter plates is described:

1. Place 1 ml of LB medium, J. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972), hereby incorporated by reference, containing an appropriate antibiotic corresponding to the antibiotic resistance marker carried on the plasmid into each of the 96 wells.

2. Inoculate bacteria containing plasmids into the wells, cover the plate with an aluminum lid, and puncture holes in the lid with a needle to allow ventilation of the wells during incubation.

3. Incubate the covered plate in a 37° shaker overnight.

4. Transfer 50 μl from each well to another 96-well microtiter plate and save for possible future use.

5. Centrifuge the original plate at 2500 x *g* for 5 minutes to pellet the cells, and decant and discard the supernates.

6. Suspend each of the cell pellets in 50 μl of deionized water by vortexing, and add 50 μl of 1% SDS in 0.2 N NaOH with shaking to mix.

7. Add 50 μl of the solution of Example 1, shake to mix, and then spin the plate at 2500 x *g* for 15 minutes to pellet cell debris, chromosomal DNA, cellular RNA, and protein, thus obtaining cleared lysates.

8. Transfer the cleared lysates to a fresh 96-well plate, mix with 50 μl of the solution of Example 1, and incubate at 37°C for 5 minutes.

9. Centrifuge the plate at 2500 x *g* for 15 minutes to pellet the plasmid DNA, and then decant and discard the supernates.

10. Wash the plasmid DNA pellets twice by suspending in 1 ml of 70% ethanol, centrifuging the plates, then decanting and discarding the supernates.

11. Dry the plasmid DNA pellets, and suspend in 50 μl of TE.

A clone of pUT626 is prepared using this procedure. The recoveries of plasmid DNA among the samples is relatively consistent, and no inter-well contamination is observed.

Example 5

A clone of plasmid pHHC (2768 bp) is isolated according to the procedures of Example 2 and sequenced by the dideoxy method, F. Sanger et al., DNA Sequencing with Chain-terminating Inhibitors, 74 Proc. Nat'l Acad. Sci. USA 5463 (1977), hereby incorporated by reference, using ^{35}S-α-dATP and "SEQUENASE" version 2.

In another preferred embodiment of the invention, a DNA isolation solution has been developed that has been successfully used for the rapid isolation of plasmid DNA from bacterial cultures. The composition comprises an effective amount of an enzyme for digesting cellular RNA, an effective amount of a stabilizer for protecting plasmid DNA from nuclease digestion, and an effective amount of a precipitating agent for precipitating plasmid DNA, as described above. A method of using the solution is simple, fast, convenient, and cost effective. More than 95% of the plasmid DNA can be recovered from a cell lysate by this method. The DNA obtained by this method is free of RNA contamination and is suitable for various manipulations in molecular cloning and DNA sequencing. The method has been adapted to a 96-well microtiter plate format for high throughput minipreps, which is very useful for screening large numbers of clones.

The present method contains at least two distinct improvements over conventional methods that also involve the use of PEG for plasmid purification. First, by the addition of a bivalent cation chelating agent, such as EDTA, in the solution used for plasmid isolation, plasmid DNA can be protected from degradation by cellular DNases during the 37°C incubation with RNase without first performing ethanol precipitation and the hazardous and tedious phenol-chloroform extraction. Secondly, by the addition of EDTA and RNase in the PEG solution, three objectives are obtained simultaneously: (1) protecting the plasmid DNA, (2) digesting the RNA, and (3) precipitating the plasmid DNA. In conventional PEG methods, these objectives are realized in three or more separate steps. Because of these improvements and simplifications, the present method is simpler, faster, and can be adapted to large-scale miniprep procedures using a 96-well microtiter plate, as described below.

The plasmid isolation solution is composed of non-toxic components and is stable at 4°C for at least 6 months. Other reagents required are TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0), 1% SDS in 0.2 N NaOH, 3 M Na acetate (pH 5.0), and 70% ethanol. For large-scale minipreps, 96-well (2 ml/well) microtiter plates (e.g. Beckman #140504) and aluminum lids (e.g. Beckman #538619) are preferred.

Example 6

In this example, an illustrative composition according to the present invention is prepared by making a solution comprising 20 μg/ml of RNase A, 20 mM EDTA, and 20% PEG8000 in deionized water.

Example 7

An illustrative embodiment of the present method of isolating plasmid DNA comprises the steps of (a) preparing an alkaline lysate of bacterial cells, (b) mixing equal volumes of lysate and the composition prepared according to Example 6, (c) incubating the mixture at 37°C for a few minutes, and (d) then centrifuging incubated mixture to pellet the plasmid DNA. Preferably, the plasmid pellet is then washed to remove residual contaminants. The washed plasmid DNA is then dried and suspended in an appropriate buffer or water.

Example 8

In this example, a miniprep procedure using the composition according to Example 6 is described:

1. Pellet 1.5 ml of overnight bacterial culture at 12,000 x *g* for 20 seconds.

2. Suspend the cell pellet in 50 μl of TE buffer by vigorous vortexing.

3. Add 80 μl of 1% SDS in 0.2 N NaOH, and mix by inverting the tube.

4. Add 80 μl of 3 M Na acetate, and mix by inverting.

5. Centrifuge the tube at 12,000 x *g* for 2 min. to pellet the cell debris.

6. Transfer the supernate to a tube pre-loaded with 200 μl of the solution of Example 6.

7. Mix by vortexing and incubate the tube at 37°C for 2 min.

8. Centrifuge at 12,000 x *g* for 2 minutes, decant and discard the supernate.

9. Wash the pellet twice by suspending it in 0.5 ml of 70% ethanol, centrifuging it to the bottom of the tube, and decanting and discarding the supernate.

10. Dry the DNA pellet, and then suspend it in 50 μl of TE.

A miniprep of plasmid pUC19 was prepared by this procedure from 1.5 ml of an overnight culture of plasmid-containing *E. coli* DH5α. Plasmid DNA was also prepared by direct ethanol precipitation from half of the same cell lysate. The purity of the plasmid DNA prepared according to the present invention was checked by agarose gel electrophoresis, and the yield (98.6%) was determined as the percent of plasmid DNA obtained by direct ethanol precipitation (FIG. 1). These results indicate that the plasmid DNA prepared according to the present invention is clean and the yield is satisfactory.

Example 9

In this example, a midiprep procedure using the composition of according to Example 6 is described:

1. Pellet 50 ml of overnight culture at 12,000 x *g* for 60 seconds.

2. Suspend the pellet in 1 ml of TE buffer by pipetting.

3. Add 3 ml of 1% SDS in 0.2 N NaOH, and mix by inverting the tube.

4. Add 3 ml of 3 M Na acetate, and mix by inverting the tube.

5. Centrifuge the tube at 12,000 x *g* for 5 minutes to pellet the cell debris.

6. Transfer the supernate to a tube pre-loaded with 7 ml of the solution of Example 6.

7. Mix by vortexing and incubate the tube at 37°C for 5 minutes.

8. Centrifuge at 12,000 x *g* for 10 minutes and decant the supernate.

9. Wash the pellet twice by suspending it in 5 ml of 70% ethanol, centrifuging it to the bottom of the tube, then decanting and discarding the supernate.

10. Dry the DNA pellet, and suspend it in 500 μl of TE.

11. Centrifuge the TE suspension at 12,000 x *g* for 1 minute and transfer the clear plasmid-containing supernate to a fresh tube.

To determine whether the plasmid DNA purified according to this procedure was suitable for digestion with restriction endonucleases, plasmid pBKBH10S (11857) was prepared from 50 ml of an overnight culture of bacterial strain STBL2. The plasmid was digested with 32 restriction enzymes at their respective optimal temperatures for 1 hour. The digested DNAs were then fractionated by electrophoresis on 0.6% agarose gel (FIG. 2). The results show that the plasmid DNA purified with the solution of Example 6 was completely digested by the enzymes tested, and there was no RNA interfering with the detection of the plasmid bands.

Example 10

In this example, a large-scale miniprep procedure using 96-well microtiter plates and the composition according to Example 6 is described:

1. Aliquot 1 ml of antibiotic-containing LB medium, J. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972), hereby incorporated by reference, into each of the 96 well using a repeat pipetter.

2. Inoculate one colony of bacteria into a well, cover the plate with an aluminum lid, and puncture holes on the lid with a needle to allow ventilation of the wells during incubation.

3. Incubate the covered plate in a 37°C shaker overnight.

4. Transfer 50 μl of the overnight culture with a multichannel pipetter to another 96-well plate and save for possible future use.

5. Centrifuge the original plate at 2500 x *g* for 5 minutes to pellet the cells, then decant and discard the supernates.

6. Suspend the cell pellet in 50 μl of TE by vortexing, then add 80 μl of 1% SDS in 0.2 N NaOH and shake to mix.

7. Add 80 μl of 3 M Na acetate, shake to mix, then spin the plate at 2500 x *g* for 15 minutes to pellet cell debris.

8. Transfer the supernates to a fresh 96-well plate, mix with one volume of the solution of Example 6, and incubate at 37°C for 5 minutes.

9. Centrifuge the plate at 2500 x *g* for 15 minutes to pellet the DNA, then decant and discard the supernates.

10. Wash the pellets twice by suspending in 1 ml of 70% ethanol, centrifuging the pellets to the bottom of the wells, then decanting and discarding the supernates.

11. Dry the DNA pellets, and suspend them each in 50 μl of TE.

A clone of plasmid pUT626 (3185 bp) was prepared using the large-scale procedure. As shown in FIG. 3, the recoveries of the plasmid among the samples were relatively consistent, and no inter-well contamination occurred during the procedure, since the wells that were not inoculated with bacteria produced no plasmid DNA.

Example 11

A clone of plasmid pHHC (2768 bp) was isolated according the procedure of Example 8 and sequenced by the dideoxy method, F. Sanger et al., DNA Sequencing with Chain-terminating Inhibitors, 74 Proc. Nat'l Acad. Sci. USA 5463 (1977), hereby incorporated by reference, using 35S-α-dATP and "SEQUENASE version 2." The plasmid purified by this method was shown to be suitable for sequencing applications.

The solution and the plasmid isolation procedures described herein have many advantages over other conventional and commercially available methods. For example, the present method is simple. In a preferred embodiment, one volume of solution is added to a bacterial cell lysate and, after a short incubation (2-5 minutes), the plasmid DNA is obtained by centrifugation. The procedure is also fast. Plasmid minipreps can be completed in 20-30 minutes. With the large-scale 96-well miniprep procedure, 96 minipreps can be obtained in about 90 minutes. Also, the procedure is safe. The solution contains no toxic components, and no steps in the procedure require toxic organic solvents. Further, the procedure is convenient. The SDS-NaOH lysis procedure is already routine in many laboratories. There is no need for glass or silica resins, columns, syringes, and manifolds. Moreover, the procedure is economical. The reagent costs for the solution is

estimated to be about 5 cents per miniprep. Only two microfuge tubes and a few pipette tips are required for each standard miniprep. The solution is easy to prepare and is a single solution product. It accompanies no other specialized reagents and accessories. The plasmid DNA obtained is clean and in good yield. There is no detectable RNA contamination, and the OD 260/280 ratio is about 1.8. More than 95% of the plasmid DNA in the cell lysate can be recovered.

CLAIMS

We claim:

1. A composition comprising at least about 3 M $MgCl_2$ and about 10% (w/v) of polyethylene glycol.

2. The composition of claim 1 wherein said polyethylene glycol has an average molecular weight of about 6000-8000.

3. The composition of claim 1 wherein said $MgCl_2$ is present in a concentration of about 3 molar.

4. A method of isolating plasmid DNA from a bacterial culture comprising the steps of:

(a) lysing bacterial cells in said bacterial culture to result in a bacterial lysate;

(b) mixing $MgCl_2$ and polyethylene glycol with said bacterial lysate to form a first mixture comprising at least about 1 M $MgCl_2$ and about 3.3% (w/v) polyethylene glycol, thereby precipitating cellular debris, chromosomal DNA, cellular RNA, and proteins in said first mixture;

(c) removing precipitated cellular debris, chromosomal DNA, cellular RNA, and proteins from said first mixture to form a cleared lysate;

(d) mixing $MgCl_2$ and polyethylene glycol with said cleared lysate to form a second mixture comprising at least about 1.5 M $MgCl_2$ and about 5% (w/v) polyethylene glycol, thereby precipitating plasmid DNA in said second mixture; and

(e) separating precipitated plasmid DNA from said second mixture to result in isolated plasmid DNA.

5. The method of claim 4 wherein step (a) comprises lysing said bacterial cells by alkaline lysis.

6. The method of claim 4 wherein said $MgCl_2$ and polyethylene glycol, prior to mixing with said bacterial lysate or said cleared lysate, are contained in a composition comprising at least about 3 M $MgCl_2$ and about 10% (w/v) polyethylene glycol.

7. The method of claim 6 wherein said composition comprises about 3 M $MgCl_2$.

8. The method of claim 6 wherein said polyethylene glycol has an average molecular weight of about 6000-8000.

9. The method of claim 4 further comprising washing the isolated plasmid DNA with a wash solution to remove residual contaminants.

10. The method of claim 9 wherein the wash solution comprises about 70% ethanol in water.

11. A method of making a cleared bacterial lysate from a bacterial culture comprising the steps of:

(a) lysing bacterial cells in said bacterial culture to result in a bacterial lysate;

(b) mixing $MgCl_2$ and polyethylene glycol with said bacterial lysate to form a mixture comprising at least about 1 M $MgCl_2$ and about 3.3% (w/v) polyethylene glycol, thereby precipitating cellular debris, chromosomal DNA, cellular RNA, and proteins in said mixture; and

(c) separating precipitated cellular debris, chromosomal DNA, cellular RNA, and proteins from said mixture to form said cleared lysate.

12. A composition for isolating plasmid DNA from bacterial cultures comprising an effective amount of an enzyme for degrading cellular RNA, an effective amount of a stabilizer for protecting plasmid DNA from degradation, and an effective amount of a precipitating agent for precipitating plasmid DNA.

13. The composition of claim 12 wherein said enzyme is ribonuclease and said effective amount of enzyme is in the range of about 1-100 μg/ml.

14. The composition of claim 13 wherein said effective amount of enzyme is in the range of about 5-50 μg/ml.

15. The composition of claim 13 wherein said stabilizer is a bivalent cation chelating agent.

16. The composition of claim 15 wherein said bivalent cation chelating agent is selected from the group consisting of ethylene diaminetetraacetic acid and salt thereof, and said effective amount of stabilizer is in the range of about 1-100 mM.

17. The composition of claim 16 wherein said effective amount of stabilizer is in the range of about 5-50 mM.

18. The composition of claim 16 wherein said precipitating agent is polyethylene glycol and said effective amount of precipitating agent is in the range of about 5-25% by weight.

19. The composition of claim 18 wherein said polyethylene glycol has an average molecular weight of about 6000-8000.

20. A method of isolating plasmid DNA from a bacterial culture comprising:

(a) lysing bacterial cells in said bacterial culture to result in a bacterial lysate and removing cellular debris, chromosomal DNA, and proteins to obtain a clarified lysate;

(b) mixing said clarified lysate with an effective amount of a composition comprising an effective amount of an enzyme for digesting cellular RNA, an effective amount of a stabilizer for protecting plasmid DNA from nuclease degradation, and an effective amount of a precipitating agent for precipitating plasmid DNA, to obtain a mixture, and incubating said mixture for a time and at a temperature sufficient for said enzyme to digest cellular RNA contained in said mixture and for said precipitating agent to precipitate plasmid DNA contained in said mixture;

(c) separating said precipitated plasmid DNA from said mixture and washing said precipitated plasmid DNA in a wash solution to obtain a DNA pellet; and

(d) drying said DNA pellet and then resuspending said DNA pellet in a selected amount of water or an aqueous buffer.

21. The method of claim 20 wherein said lysing comprises alkaline lysis.

22. The method of claim 21 wherein said removing cellular debris, chromosomal DNA, and proteins to obtain a clarified lysate comprises salt precipitation.

23. The method of claim 20 wherein said enzyme is ribonuclease and said effective amount of enzyme is in the range of about 1-100 μg/ml.

24. The method of claim 23 wherein said effective amount of enzyme is in the range of about 5-50 μg/ml.

25. The method of claim 20 wherein said stabilizer is a bivalent cation chelating agent.

26. The method of claim 25 wherein said bivalent cation chelating agent is selected from the group consisting of ethylene diaminetetraacetic acid and salts thereof, and said effective amount of stabilizer is in the range of about 1-100 mM.

27. The method of claim 26 wherein said effective amount of stabilizer is in the range of about 5-50 mM.

28. The method of claim 20 wherein said precipitating agent is polyethylene glycol and said effective amount of precipitating agent is in the range of about 5-25% by weight.

29. The method of claim 28 wherein said polyethylene glycol has an average molecular weight of about 6000-8000.

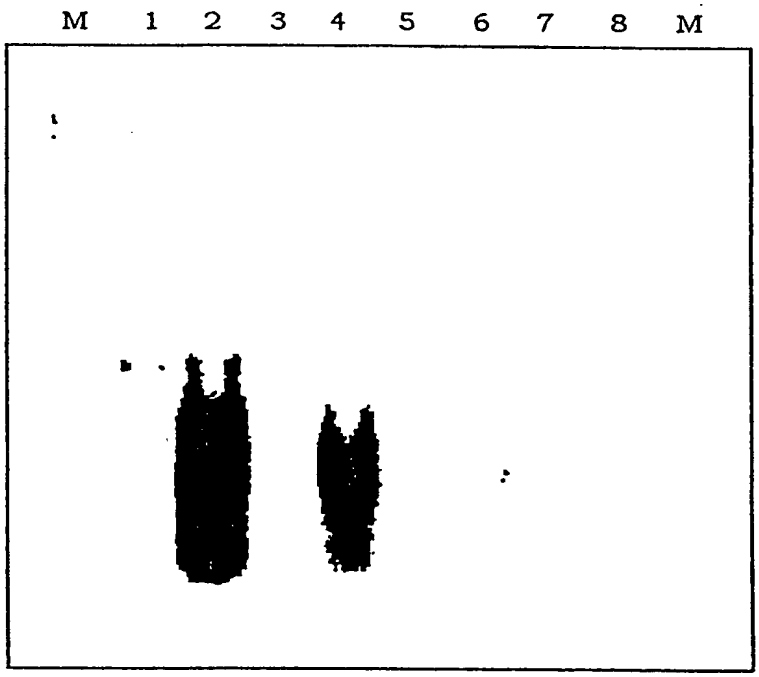

Fig. 1.

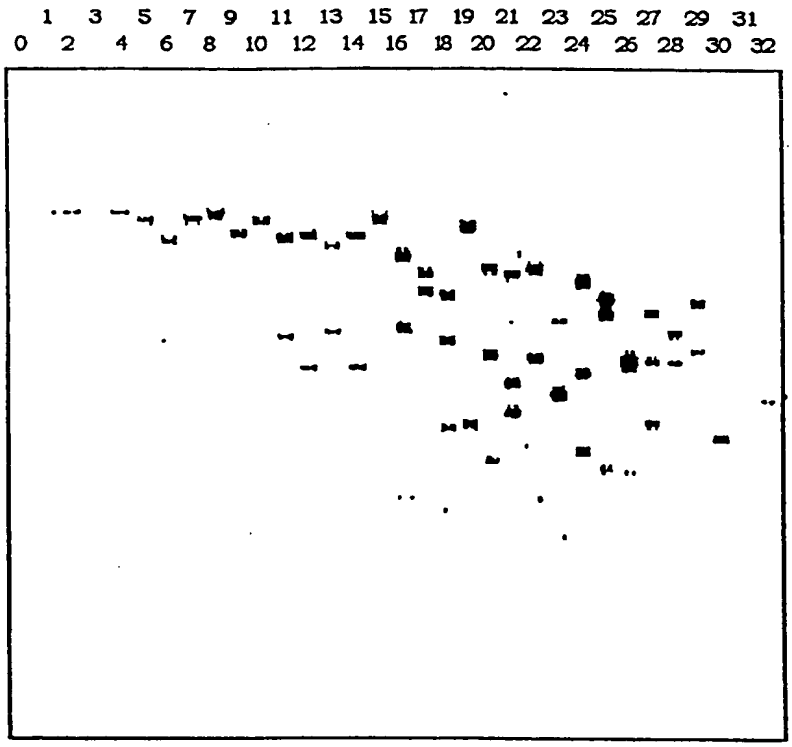

Fig. 2.

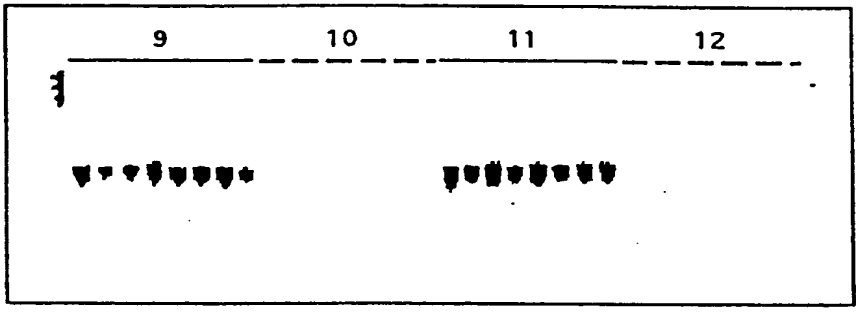

Fig. 3.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18762

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 19/34
US CL :536/25.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/101, 262, 267, 270; 536/25.3, 25.4, 25.41, 25.42; 935/16, 19, 59

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E,X --- E,Y	US, 5,705,628 A (HAWKINS) 06 January 1998, see column 5, lines 29-65, and especially column 2, line 16, through column 8, line 51.	1-3 --- 4-11
Y	US 5,561,064 A (MARQUET ET AL.) 01 October 1996, see especially column 10, line 41, through column 11, line 8.	1-11
A	Matthews et al., "REVIEW, Analytical Strategies for the Use of DNA Probes", Analytical Biochemistry, February 1988, Volume 169, pages 1-25, see especially pages 4-5 at the sections entitled "2.2 Preparation of Restrictable DNA" and "2.3 Rapid Procedures".	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
01 FEBRUARY 1998

Date of mailing of the international search report
20 FEB 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18762

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Maniatis et al., MOLECULAR CLONING, A LABORATORY MANUAL, 1982, (Published by the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA), pages 80-82.	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18762

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18762

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG covering CAS, EMBASE, WPI, BIOTECH ABSTRACTS, and MEDLINE with search terms: PEG, polyethylene, glycol, lysis, lysate, lyse, precipitate

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-11, drawn to a composition including magnesium chloride and polyethylene glycol and methods of its use.

Group II, claim(s) 12-29, drawn to a composition including an enzyme for degrading RNA, a stabilizer, and a precipitating agent and methods for its use.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is directed to minimally requiring a magnesium salt with polyethylene glycol whereas Group II minimally requires an enzyme, a stabilizer, and a precipitating agent. Thus, each group requires a different combination of materials and therefore neither share the same nor corresponding special technical features.